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# EXHIBIT

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**WORKING GROUP MEETING:****Technologies for *in situ* Repair of Single Nucleotide Mutations in Single Gene-Defect Blood Diseases**

Monday, September 24, 2001, 8:30 AM- 4 PM  
Rockledge II Building  
6701 Rockledge Dr. Room 9104  
Bethesda, MD  
National Institutes of Health

Sponsored by  
The National Heart, Lung, and Blood Institute

**AGENDA**

- 8:30 am **Welcome**  
Greg Evans, Ph.D., Blood Diseases Program, NHLBI
- 8:40 am **Efforts to Develop Psoralen-Oligonucleotide Third Strands for Reversing Substitution Mutations**  
Jacques Fresco, Ph.D., Princeton University
- 9:10 am **Targeted Genome Modification Via Triple Helix Formation**  
Peter Glazer, MD, Ph.D., Yale University
- 9:40 am **Gene Repair by Oligonucleotides: Treatment of Skin Disorders**  
Kyongseun Yoon, Ph.D., Jefferson Medical College
- 10:10 am **Break**
- 10:25 am **Repair of Single Nucleotide Mutations In Single Gene-Defect Blood Diseases**  
Cliff Steer, M.D., University of Minnesota
- 10:55 am **Sequence-Specific Modification of the Human  $\beta$ -Globin Gene by Small Fragment Homologous Replacement (SFHR)**  
Dieter Gruenert, Ph.D., University of Vermont
- 11:25 am **Delivery of Oligonucleotides In Cell Culture and *In vivo*.**  
Ryszard Kole, Ph.D., University of North Carolina, Chapel Hill
- 11:55 am **Lunch**
- 12:55 pm **Repair of Genetic Instructions by Ribozymes**  
Bruce Sullenger, Ph.D., Duke University
- 1:25 pm **Title to be announced**  
Don Miller, MD, Ph.D., University of Louisville
- 1:55 pm **Break**
- 2:05 pm **Group Discussion of Key Topics for Future Focus in Gene Correction Research**  
Greg Evans, Ph.D., NHLBI
- 4:00 pm **ADJOURNMENT**

## SEQUENCE-SPECIFIC MODIFICATION OF THE HUMAN $\beta$ -GLOBIN GENE BY SMALL FRAGMENT HOMOLOGOUS REPLACEMENT (SFHR)

Goncz KK<sup>1</sup>, Prokopishyn N<sup>2</sup>, Chow B<sup>2</sup>, Davis BR<sup>2</sup>, Gruenert DC<sup>1</sup>. <sup>1</sup>Human Molecular Genetics Unit, Department of Medicine, University of Vermont, Burlington VT, USA <sup>2</sup>Gene-Cell, Inc., Houston TX, USA

The plethora of hemoglobinopathies with a genetic etiology have been elusive in terms of their response to conventional therapies. The development of genetic therapies has provided new possibilities for treatment of these disorders. While conventional cDNA-based gene therapy has shown some success, there are still numerous limitations in using these strategies as an effective therapeutic modality for inherited diseases. As an alternative to the cDNA-based gene therapy approaches, a number of groups have developed and implemented strategies that directly repair/modify mutant lesions within the genetic DNA. One strategy, small fragment homologous replacement (SFHR), has already been demonstrated to be effective at the simultaneous modification of multiple bases within selectable marker genes (Zeocin) and within the cystic fibrosis transmembrane conductance regulator (CFTR) gene, both *in vitro* and *in vivo*. More recently, SFHR has been applied to site-specific modification of the human  $\beta$ -globin gene at the genetic locus responsible for sickle cell anemia. Studies were carried out in murine leukemia (MEL) cell lines carrying human chromosome 11 and in isolated human hematopoietic stem/progenitor cells that were CD34<sup>+</sup> CD38<sup>-</sup> lin<sup>-</sup> as well as human bronchial epithelial cells. The cells were transfected, either with DNA lipid complexes (MEL cells) or by direct nuclear microinjection of DNA fragments (human hematopoietic stem/progenitor cells). The fragment used contained the  $\beta^s$ -globin mutation. After transfection, cells were analyzed either by allele-specific and nonallele-specific DNA polymerase chain reaction (PCR) amplification or RNA reverse transcriptase PCR (RT-PCR) amplification. Nonallele-specific amplification was followed by restriction fragment length polymorphic (RFLP) analysis using the Dde I restriction enzyme. Analysis of the cells indicated that the wild-type  $\beta^A$ -globin had been replaced by the targeting fragment sequences containing the sickle cell mutation (A→T transversion at codon 6). SFHR-mediated modification was observed in the MEL cells as well as the human bronchial epithelial cell line that did not express  $\beta$ -globin following transfection with a lipid-DNA complex. Microinjection experiments in the human stem/progenitor cells showed stable SFHR-mediated modification. The microinjection experiments were particularly effective for assessing the effect of fragment number on replacement fragment concentration. No replacement was observed at fragment concentrations of < 1000 fragments/fl with an injection volume of 3-5 fl/cell. Replacement was consistently observed at fragment concentrations of 1000 fragments/fl to 5000 fragments/fl. Experiments with human hematopoietic stem/progenitor cells showed that SFHR-mediated modification was stable with continuous growth up to at least 5 weeks. Sequence analysis of a PCR amplicon generated with primers outside the homologous region indicated that, in one experiment up to 70% of the alleles in the cells of the outgrowth population had undergone homologous replacement. In this experiment, 65% of the cells were microinjected and grown for 3 weeks. These studies demonstrate that SFHR-mediated modification can occur in hematopoietic cells at the  $\beta$ -globin gene locus and that transcription is not necessary for homologous replacement (airway epithelial transfection results). These findings are encouraging for the further development of SFHR as a genetic therapy for hemoglobinopathies. These studies were supported, in part, by NIH grant DK53925 and by funds from Gene-Cell, Inc.

Table 2. PCR and *XhoI* restriction digestion fragments

Primer	Fragment size (bp)
Non-allele-specific DNA	
CF1/CF5 <sup>a</sup>	491 (N)
	488 (ΔF)
CF1B/CF6 <sup>b</sup>	771 (N)
	768 (ΔF)
Allele-specific DNA	
CF1B/CF7C <sup>b</sup>	392 (N)
<i>XhoI</i>	283/109
CF1B/CF8C <sup>b</sup>	389 (ΔF)
<i>XhoI</i>	283/106
CF1/CF7C <sup>c</sup>	308 (N)
<i>XhoI</i>	199/109
CF1/CF8C <sup>c</sup>	305 (ΔF)
<i>XhoI</i>	199/106
CF7B/CF6 <sup>b</sup>	414 (N)
CF8B/CF6 <sup>b</sup>	411 (ΔF)
Allele-specific RNA	
CF17/CF7C <sup>d</sup>	330 (N)
<i>XhoI</i>	221/109
CF17/CF8C <sup>d</sup>	327 (ΔF)
<i>XhoI</i>	221/106

Different primer pairs and the resulting fragments following amplification and digestion with *XhoI*. (N), normal genotype; (ΔF), ΔF508 genotype. Conditions for the specific primer pairs were as follows.

<sup>a</sup>Primers 0.4 μM, DNA 50–100 ng, Mg<sup>2+</sup> 1.5 mM; 94°C for 60 s, denaturation; 55°C for 30 s, annealing; 72°C for 30 s with 4 s/cycle increase, extension: 30 cycles.

<sup>b</sup>Primers 0.5 μM, DNA 50–100 ng, Mg<sup>2+</sup> 2.0 mM; 95°C for 60 s, denaturation; 59°C for 60 s, annealing; 72°C for 90 s, extension: 35 cycles with 8 min extension on the last cycle.

<sup>c</sup>Primers 0.5 μM, DNA 50–100 ng, Mg<sup>2+</sup> 2.0 mM; 95°C for 2 min, denaturation; 57°C for 60 s, annealing; 72°C for 90 s, extension: 35 cycles with 8 min extension on the last cycle.

<sup>d</sup>Primers 1.0 μM; DNA 50–100 ng, Mg<sup>2+</sup> 2.0 mM; 94°C for 30 s, denaturation; 57°C for 30 s, annealing; 72°C for 30 s, extension: 35 cycles with 8 min extension on the last cycle.

### Transfection of DNA

Cells were transfected with either the 491 or 488 nt fragments complexed with either the polyamidoamine cascade polymer Starburst dendrimer (13,34) or with cationic lipid (Lipofectamine; Gibco BRL, Grand Island, NY). Dendrimer–DNA complexes were prepared as described previously (13). Complexes with Lipofectamine were made according to the manufacturer's instructions.

Cells were grown in FN/V/BSA-coated T25 tissue culture flasks to ~80% confluence (~10<sup>6</sup> cells). Prior to transfection, the complete medium was aspirated and replaced with 1.5 ml serum-free medium. One of the DNA complexes (4 μg DNA/flask) was then added to the flask and incubated for 5 h at 37°C. The transfection medium was then aspirated and replaced with

complete growth medium. Cells were cultured at 37°C after transfection, with daily replacement of medium until the cells were used for DNA and RNA isolation.

### DNA and RNA analysis

Genomic DNA and cytoplasmic RNA were isolated and purified from cells (13) 2–3 days following exposure to the fragment. Cellular DNA was analyzed using both non-allele-specific primers CF1B/CF6 (Table 1) and allele-specific primers [e.g. CF1/CF7C or 8C, CF1B/CF7C(8C) or CF7B(8B)/CF6]. Primers CF1B (sense) and CF6 (antisense) are outside the 5' and 3' ends of the homologous region, respectively, and amplification of DNA from transfected cells gives rise to a mixed population of wild-type and ΔF508 PCR products. Because these primers are also outside the homologous region defined by the template DNA in the pTA vectors, the fragment generated by CF1B/CF6 amplification will not detect unincorporated, randomly integrated fragment or residual template DNA. Allele-specific PCR analysis with primers CF7C and CF8C or CF7B and CF8B (Table 1 and 2) enables differential detection of either wild-type or ΔF508 *CFTR* alleles. CF7B (sense) and CF7C (antisense) are specific for the wt*CFTR* allele, while CF8B (sense) and CF8C (antisense) are specific for the ΔF508 *CFTR* allele. After amplification with CF7C or CF8C, PCR products can also be digested with *XhoI* enzyme as a secondary assessment of SFHR.

Cytoplasmic RNA was analyzed for the presence of ΔF508 *CFTR* mRNA sequences by reverse transcription (RT) and subsequent PCR amplification (RT–PCR). Briefly, RNA was denatured at 95°C for 2 min, then reverse transcribed. First strand cDNA was amplified using primer CF17 (located in exon 9) and allele-specific primer CF7C (N) or CF8C (ΔF508) (Table 2). PCR products from RT–PCR were also digested with *XhoI*.

PCR amplification product sizes and the conditions for RNA and DNA analyses are as indicated (Table 2). DNA fragments were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

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